Kinetics of coagulation and immunological processes

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13. Kinetics of Coagulation and Immunological Processes

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Inhibition is one of the most important phenomena observed when the presence of antibodies complicates the coagulation process. Since study of the inhibition of an enzymatic process requires the use of enzyme kinetics, a discussion of the kinetics of coagulation and immunological processes would be appropriate for this conference, if it did not involve a very difficult problem. It would be relatively easy to give a detailed treatment of the specific kinetic problems encountered in coagulation and immunology, but not without touching on certain mathematical problems, that do not lend themselves to a brief oral presentation. I therefore thought it might be more useful to discuss the kinetic approach in general and to try to reach some conclusions as to how kinetics can best be applied in the field we are interested in.

The study of coagulation, like any other kind of biochemical research, can be done via two different pathways: one tries to separate the enzymes involved and studies the separate steps of the reaction, *or* one studies the kinetics of a large part of the reaction process. This means: one extracts information from the functional reaction of the system to independently variable experimental circumstances.

These two approaches are complementary. They are used in combination in any kind of enzymological research. Yet they are of unequal status. Separation is looked upon much more favorably than kinetics, no doubt because the final stabilization of our insights is most often determined by the level achieved in the purification of the reactants involved. But it is usually kinetics which leads us when we are exploring a new field and tells us where to look for the next pure enzyme, and it is kinetics as well that finally characterizes the functions of the isolated entities. This is illustrated for instance by recent developments in the field of allosteric interactions (6).

A kinetic analysis involves a number of separate steps. *First*, a reaction mechanism is postulated in terms of a set of chemical reaction equations. *Second*, two sets of mathematical formulas are derived from these reaction equations, namely the *velocity equations* that relate the rate of change of the concentration of each of the reactants to the reaction constants and the concentrations present at a given mo-

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ment, and the *conservation equations* that relate the quantity of reactants present at zero time to the concentrations present at a given moment.

The simplest model of enzymatic action can serve as an illustration. The chemical formulas are:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} C$$

$$C \xrightarrow{k_{+2}} E + P$$

The velocity equations are:

$$dS/dt = -k_{+1} \cdot E \cdot S + k_{-1} \cdot C$$

$$dC/dt(= -dE/dt) = k_{+1} E \cdot S - (k_{-1} + k_{+2})C$$

$$dP/dt = k_{+2} \cdot C$$

The conservation equations are:

$$E_o = E + C$$

$$S_o = S + C + P$$

After these sets of equations have been set up, the *third step* is to solve them. It is here that the mathematician enters the scene. Even in the simple case given as an illustration he will not be able to solve the equations without introducing simplifying assumptions. When we accept the simplifications $S_o \gg E_o$ and $S \gg P$ for instance, the well known »Michaelis-Menten« formula will result as a solution of the above equations; i.e.

$$dP/dt = \frac{k_{+2}ES}{K_m + S} \quad \left(\text{where } K_m = \frac{k_{-1}+k_{+2}}{k_{+1}}\right).$$

The *fourth step* is to check the theoretical predictions against the experimental observations.

When the fit between these two is close enough, the only conclusion that is allowed, is that both the proposed reaction mechanism and the assumptions used to solve the equations are not contradicted by the experimental results. It is inherent to the method that it can never prove a hypothetical reaction mechanism to be valid. The most that can be achieved is to disprove alternative mechanisms and to find the simplest reaction scheme that will account for the experimental observations. Sometimes alternative schemes are possible, and a good kinetic analysis should indicate such alternatives and thereby lead to further experiments.

Now, these considerations are so basic that it hardly seems justified to mention them at this conference. Everybody knows that in the handbooks on enzymology

solutions are presented pertinent to a series of reaction schemes for example in the cases of competitive and non-competitive inhibition [comp. ref. (2)]. By using these solutions, one can bypass steps 1, 2, and 3 and start directly with checking the results of experiments against standard formulas.

The use of ready-made solutions, however, not only makes it possible to bypass a lot of work, but also avoids many considerations as to what one is really doing. The simplifications that the mathematician must introduce to solve the equations require close supervision by the biochemist, especially when we are working with non-standard systems as they occur in coagulation and immunology.

The main conclusion that presents itself is therefore, that every attempt at kinetics should go through all the steps mentioned. I will attempt to illustrate this on the basis of a few specific examples.

My first example (4) is the old question of whether a given coagulation factor acts as a substrate or as an enzyme. When one applies the Michaelis-Menten formula directly one expects that the reaction velocity will vary hyperbolically with the concentration of a substrate. This kind of behaviour is entirely dependent upon the assumed condition of a relative excess of substrate. It is easy to calculate that with reversal of this assumption (that is $E_o \gg S_o$ instead of $S_o \gg E_o$) the kinetic roles of enzyme and substrate are reversed. The role that we assign almost automatically to the substrate is in reality the role of that reactant present in excess. Because nothing can be said a priori about the relative concentrations of reactants in coagulation processes, any distinction between enzyme and substrate made on a traditional kinetic basis therefore seems unjustified.

A second example (3) can be taken from the kinetics of prothrombinase formation. Prothrombinase activity is hyperbolically related to phospholipid concentration over a large range of concentrations. In terms of traditional kinetics this puts the phospholipid in the role of a substrate. As I have already mentioned, a catalytic role is equally feasible. The situation becomes still more complicated when one tries to account for the fact that high concentrations of phospholipid have an inhibitory effect. The observed relations between phospholipid concentration and prothrombinase activity follow directly, however, when one starts from the reaction equations:

$$\begin{aligned} X_a + Ph \cdot lip &\rightleftarrows X_a\text{-}Ph \cdot lip \\ V_a + Ph \cdot lip &\rightleftarrows V_a\text{-}Ph \cdot lip \\ X_a\text{-}Ph \cdot lip + V_a &\rightleftarrows \text{prothrombinase} \\ V_a\text{-}Ph \cdot lip + X_a &\rightleftarrows \text{prothrombinase} \end{aligned}$$

The hyperbolic relationship is a consequence of the reversibility of the adsorption of the proteins onto the phospholipid and the inhibitory effect is due to the fact that factors V_a and X_a have less chance to meet when a large adsorbing surface

is offered. Here again, direct application of existing formulas would have been misleading rather than helpful.

When the inhibitory effect of an antibody is to be evaluated there is again no other way available except to start with a circumscript postulate on the molecular interactions and work out the kinetics from there on. Therefore, in a kinetic analysis the most important considerations are those on molecular interactions rather than those on concentration-velocity relationships and the like.

At first glance, the differences between coagulation processes and immunological reactions seem to be quite large. Coagulation is essentially a process of enzymatic zymogen activation, whereas antigen-antibody interaction is the formation of a complex between two or more large molecules. On further consideration, however, one realizes that the first reaction in an enzymatic interaction is the formation of a complex between substrate and enzyme. When the substrate is a protein, as is the case in blood coagulation reactions, there is no essential difference between the first stage of an enzymatic reaction and the formation of an antigen-antibody complex. The chemical forces involved must be of the same type: Coulomb forces, v. d. Waals forces, hydrogen bonding and hydrophobic interaction. The specificity of the reaction is equally impressive in both systems and must be explained in both systems by the complementary pattern of interacting groups rather than by the specificity of the type of physico-chemical bonding.

The difference between immunological reactions and enzymatic reactions thus resides in the second step. After formation of an enzyme-substrate complex, profound changes occur in the chemical binding pattern within the complex. These changes involve shifts in covalent bonds within the product. The enzyme remains essentially unmodified. No covalent alterations occur in antigen-antibody complexes, but secondary reactions do occur and so may bring about precipitation, inactivation of the antigen and so forth, or they may initiate a further set of reactions such as complement fixation, cell lysis and so on. The fact that covalent changes do occur in enzymatic reactions but not in immunological ones does not necessarily serve to distinguish the two reactions on kinetic grounds. It results in differences in changes in free energy, enthalpy and entropy; but due to the complexity of the molecules there are a multitude of changes in the so-called weak forces which can easily mask the thermodynamic effect of the breaking of covalent bonds. In any case these are thermodynamic values that are not recognized when kinetic measurements are carried out at one temperature only.

The other way of distinguishing between these two types of reactions is from the fact that unmodified enzyme is recovered after a catalytic cycle in an enzymatic reaction. This is an essential difference, yet it is not necessarily very useful in the study of coagulation reactions because there are several pathways along which clotting-enzymes can be rapidly inactivated.

The nature of the secondary reaction occurring after the formation of an antigen-antibody complex is determined by the type of antibody. The antibodies will in any case be directed against specific groups of the antigen molecule, but not necessarily against those parts of the molecule that carry its enzymatic activity. Against what part of a coagulation factor an antibody did develop its specificity remains entirely a matter of chance. So after the primary reaction the active site of the antigen part of the antigen-antibody complex may remain completely functional, have a changed function, or be completely inhibited. Then, in a secondary reaction the antigen-antibody complex that initially had its activity preserved may alter and again loose its activity. A precipitating antibody, for instance, will remove molecules from the solution; but more subtile changes can occur too. It is also possible for instance, that combination of an antibody and a coagulation factor causes quaternary constraints in the latter molecule. This may lead to a distortion of the tertiary structure surrounding the active part of the molecule and thus influence its activity even though the active site as such does not take part in the bonding with the antibody. The slow inactivation by antibodies as described by Denson for instance (1) may well be due to a reaction of this type.

In this context it is interesting to note that quaternary constraints brought about by protein-protein interaction can lead to activation as well as to inactivation. This type of activation is seen in the interaction between staphylocoagulase and prothrombin. Staphylocoagulase does not convert prothrombin into thrombin enzymatically. In all probability, staphylocoagulase complexes with prothrombin and causes distortion in the zymogen, so that a site with thrombin activity is generated in the complex (ref. 7, 8).

The possibility of the existance of antibodies directed not against protein factors but against lipoid structures with a functional role in coagulation should be recognized, too. We already have preliminary evidence that certain types of antithromboplastins act by binding not with coagulation factors, but with the hydrophobic surfaces required for the formation of active complexes of coagulation factors. It seems as though there is even a kind of specificity here. Some antithromboplastins prevent the formation of prothrombinase, that is the factor X-factor V-phospholipid complex. Others act one stage higher and interfere with the formation of the factor IX-factor X-phospholipid cluster (5).

Therefore, the effect of antibodies on a coagulation system by no means has to consist of the simple titration of a specific factor. The following alternative possibilities must be taken into consideration:

1. One can expect effects on the number of active molecules available, not only because of titration, but also because of the establishment of an equilibrium that can shift when molecules are occupied in the coagulation process.

2. There are the effects on the mode of action of the antigen. Coagulation factors bound by antibodies can have an altered but persisting activity.
3. There are the slow effects of quaternary constraints that can give rise to a slow alteration of the properties of the complex.
4. The antibodies can react not only with proteins but also for example with the phospholipids necessary for the proteins to be able to function.

Each of these effects will be superimposed upon the coagulation reactions, and the latter need not follow orthodox kinetic laws either. So in concluding, I can only repeat that in these complicated systems it is hardly useful to perform kinetic analysis by applying standard rules and formulas, even when these seem to be obeyed by the experimental data. Rather, one should postulate a reaction mechanism in terms of moleculer interactions, and then calculate the expected kinetic behaviour, exercising due caution with respect to the simplifications required for the solution of the mathematical problems. Alternative reaction schemes should be considered as well, and comparison of the results will suggest discriminative experiments. This is about the best one can hope for from the kinetic approach to problems of antigens in the biochemistry of blood coagulation.

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